

Powder Puff Spiroplasma: A New Epiphytic Mycoplasma

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Abstract. A spiroplasma (strain PPS1) isolated from healthy flowers of *Calliandra haematocephala* in Florida has been found to be a member of a serogroup of the Spiroplasmataceae. It is distinct from *Spiroplasma citri* and from other described spiroplasmas as determined by growth inhibition, fluorescent antibody, and ELISA serological tests. PPS1 was also distinguished from *S. citri* and several other spiroplasmas by the guanine + cytosine content of its DNA. PPS1 requires sterol for growth, is inhibited by digitonin, grows at 20–30°C, and does not hydrolyze arginine or urea. The ready isolation of this and similar organisms from surfaces of healthy plants emphasizes that caution should be exercised in attempts to isolate cell wall-less prokaryotes from the interior of diseased plants. Although some strains of spiroplasmas are known as insect pathogens in nature, the ecological role(s) of the flower-inhabiting spiroplasmas has yet to be fully determined.

Introduction

The reports by Davis et al. [7, 13] on the isolation of spiroplasmas and non-helical mycoplasmas from healthy tulip tree flowers in Maryland and Connecticut were the first to suggest that these fastidious, wall-free prokaryotes may not be limited in their habitats to arthropods and the phloem of diseased plants, as previously considered. Their findings were substantiated by the isolation by McCoy et al. [24] of several helical and nonhelical mycoplasmas from surfaces of tropical flowers in Florida and by isolation of spiroplasmas from flowers elsewhere [5, 8, 27, 33]. One of the organisms isolated in Florida was a spiroplasma obtained from flowers of the powder puff plant (*Calliandra haematocephala* Hassk.) and designated PPS1. Results of the initial characterization of this organism are presented here.

Materials and Methods

Isolation

The PPS1 spiroplasma was isolated from healthy powder puff flowers in southern Florida. Flowers were collected in the field without being touched by hand, transported to the laboratory in plastic

Table 1. Spiroplasma isolates used in comparative tests with strain PPS1 isolated from flowers of *Calliandra haematocephala* in Florida

Strain	Original isolation		Culture medium	Serogroup	
	Source	Location		Davis et al. [11]	Junca et al. [18]
<i>S. citri</i> (SC-27)	Citrus	California	MC	IA	I.1
Honey bee (G1)	Spanish needle flower	Florida	MC	IB	I.2
Corn stunt spiroplasma (CSO) (B-2)	Maize	Mexico	MC	IC	I.3
<i>S. floricola</i> (23-6)	Tulip tree flower	Maryland	MC	II	III
Spiroplasma (SR3)	Tulip tree flower	Connecticut	MC	III	IV
<i>S. mirum</i> (SMCA)	Rabbit tick	Georgia	SP-4	IV	V

bags, removed, and rinsed briefly in SP-4 mycoplasma culture broth [3] without prior surface sterilization. This broth was immediately passed through a sterile 0.45 μm pore diameter filter to remove bacteria and other nonfilterable microorganisms and debris, and the filtrate was used to inoculate tubes of sterile broth. Tubes were incubated at 30°C and observed for a color change of the phenol red dye incorporated in the medium as an indicator of growth. Tubes showing a color change from red to yellow were examined for the presence of microorganisms by phase contrast microscopy at 1,000 \times . Some cultures contained helical mycoplasmas (spiroplasmas); others contained nonhelical mycoplasmas, as noted previously [24]. Some tubes containing spiroplasmas or nonhelical mycoplasmas also contained bacteria. Such cultures could be freed of bacteria by filtration through a 0.45 μm pore diameter filter and passage of the filtrate into sterile fresh broth. An isolate representative of the cultured spiroplasmas was called strain PPS1 and was studied further.

Following primary isolation, the PPS1 spiroplasma was subcultured 3 times and frozen in a REVCO* ultralow temperature freezer at -40°C. The isolate was then triple cloned [2] to ensure purity of the strain used in further characterization tests [1]. The cloned PPS1 strain has been deposited with the American Type Culture Collection and has their accession designation ATCC 33450. Other spiroplasmas used in comparative studies are listed in Table 1.

Growth Studies

The PPS1 spiroplasma was cultured in a number of media, including SP-4, LD 8 [2, 20], C3-G [22], and MC (PPLO broth plus 4% sucrose, 20% horse serum, and 2.5% fresh yeast extract obtained from Grand Island Biological Co., Grand Island, NY). Growth at 20, 30, 37, and 40°C was assessed by observing color change in broth, and was confirmed by direct observation of organism helices under dark field at 1,000 \times . The multiplication rates of the PPS1 spiroplasma were determined both by counting helices in a droplet on a microscope slide by dark field microscopy, as described previously [23], and by dilution tube assay in which serial 10-fold dilutions of the culture were made, and numbers of color changing units (CCU) per ml in the initial tube were estimated from the limiting dilution in which growth occurred.

The sterol requirement of PPS1 was determined by assessing growth after serial culture through 6 passages of serum-free SP-4 broth and by inhibition of growth by digitonin, a specific binder of

* Mention of a commercial company or specific equipment does not constitute its endorsement by the University of Florida or the U.S. Department of Agriculture over similar equipment or companies not named.

Table 2. Digitonin inhibition, arginine utilization, and oxytetracycline (OTC) inhibition of PPS1 spiroplasma compared with other spiroplasmas and with *Mycoplasma gallisepticum* and *Acholeplasma laidlawii*

Strain ^a	Digitonin inhibition zone (mm)	Arginine utilization	Minimum inhibitory OTC concentration (μg/ml)
PPS1	7.0	No	0.041
<i>S. citri</i> (SC-27)	14.1	Yes	0.041
G1	8.5	Yes	0.041
CSO strain B-2	ND ^b	Yes	0.013
SR3	10.7	No	0.041
<i>S. floricola</i> (23-6)	7.5	Yes	0.370
<i>M. gallisepticum</i>	7.5	ND	ND
<i>A. laidlawii</i>	0	ND	ND

^a Strain abbreviations same as Table 1.

^b ND = not done.

sterol. Digitonin inhibition was determined according to the method of Freundt et al. [16] by placing a 6 mm diameter paper disk, soaked in a 1.5% ethanolic solution of digitonin and air dried, on a flood-inoculated agar surface. The widths of the growth inhibition zones were measured after colony development and compared to those obtained with known sterol requiring and non-requiring mycoplasmas.

The ability of PPS1 to hydrolyze arginine was determined in microtiter plates by assessing pH change in MC broth containing 0, 12.5, and 25 mM supplemental arginine. Arginine hydrolysis in positive control cultures resulted in a rise in pH, indicated by an intensification of the red coloration of the phenol red dye incorporated in the medium. Plates were observed every 8–12 h and the time required for any increase in pH was recorded. Observations continued for 10 times the length of time required for a pH decrease (indicative of hydrolysis of sugar) to occur in the inoculated medium unsupplemented with arginine. Each measurement was replicated 8 times and the experiment was repeated twice. The arginine hydrolytic activity of PPS1 was compared to that of *Spiroplasma citri* strain SC27; honey bee spiroplasma strain G1 [8]; corn stunt spiroplasma strain B-2; and flower spiroplasmas SR3 and *S. floricola* (strain 23-6) [7, 12] (Table 2).

The ability of oxytetracycline-HCl (OTC) to inhibit the growth of the same group of organisms used in the arginine hydrolysis test was also measured. OTC concentrations ranged from 0.004–30 μg/ml MC medium, prepared in a 3-fold dilution series using microtiter plates. The lowest concentration of OTC at which growth had not occurred after 20 days was recorded as the minimum inhibitory concentration (MIC) for each organism.

Serological Characterization

Antisera specific for spiroplasmas were prepared by standard methods using rabbits inoculated with phosphate-buffered saline (PBS)-washed spiroplasmas emulsified in Freund's incomplete adjuvant. Antiserum to *S. mirum* was provided by J. Tully (National Institutes of Health, Bethesda, MD 20205).

Growth inhibition assays [6] were performed on the surface of SP-4 agar in 60 mm petri plates. Drops (10 μl) of 10⁻⁴, 10⁻⁵, and 10⁻⁶ dilutions of actively growing cultures of the test organisms were allowed to run across the surface of an agar plate by tilting the plate, and the resulting streaks were allowed to dry. Six mm diameter paper disks dipped in appropriate antisera and allowed to

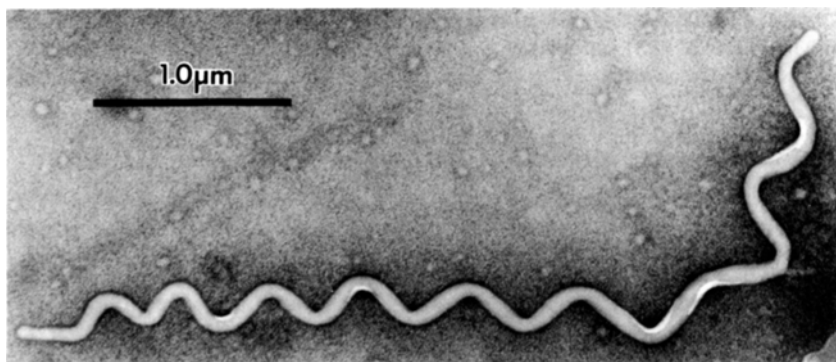


Fig. 1. Transmission electron micrograph of the PPS1 spiroplasma contrasted with ammonium molybdate.

dry were then placed in the center of each streak. After appropriate incubation periods at 30°C, the widths of the zones of growth inhibition outside the disks were measured.

Spiroplasma serological deformation titers were taken using PPS1 antiserum in a 3× dilution series, as described by Williamson et al. [34].

Fluorescein conjugates [3] were prepared with antisera specific for spiroplasma strains PPS1, G1, SC-27, SR3, and 23-6. Droplets of conjugated sera diluted with PBS were incubated for 30 min at 37°C on the surface of actively growing cultures of the organisms in petri plates containing MC agar. After the plates were rinsed with PBS, the treated area was shaved from the surface of the agar with a sharpened spatula, and the specimen was observed for fluorescence by dark field microscopy on a Zeiss microscope with FITC filters. Intensity of fluorescence was visually estimated.

Enzyme-linked immunosorbent assays (ELISA) were performed in polystyrene microtiter plates as per Clark and Adams [4]. Plates were coated with γ -globulin (4.0 μ g/well) prepared from specific antisera. Antigen samples consisting of PBS-washed spiroplasmas (2.5 μ g protein/well) were placed in coated plates and incubated overnight at 4°C. Plates were rinsed and alkaline phosphatase conjugated antiserum, prepared from the coating γ -globulin, was incubated in the sample wells for 4 h at 37°C. The amount of enzyme-conjugated antiserum bound to the spiroplasma antigen on the surface of the wells was determined by incubation of p-nitrophenyl phosphate substrate for the alkaline phosphatase in the sample wells for 30–60 min at 37°C. The amount of substrate hydrolyzed was recorded as increased absorbance (at 405 nm) in the substrate solution.

PPS1 conjugate reacted with uninoculated MC medium in preliminary ELISA experiments. This activity was eliminated by affinity chromatography of the conjugated antibody. The affinity gel was prepared by conjugation of protein precipitated from MC medium saturated with ammonium sulfate with Sepharose 4CBP (Pharmacia Fine Chemicals, Piscataway, NJ 08854). Conjugation and chromatography were according to the manufacturer's directions.

Guanine + Cytosine Content of DNA

The base composition of deoxyribonucleic acid (DNA) from spiroplasma strain PPS1 was estimated by thermal denaturation of double-stranded DNA in 10 mM sodium phosphate (pH 7.9) in 1 mM EDTA, using a Gilford Model 2400 recording spectrophotometer with temperature programmer [21]. DNA was prepared by a method noted previously [21] from 1 liter of culture grown 24 h in liquid medium LD8 at 31°C. DNA from *S. citri* strain Maroc R8A2 was included as a standard. The guanine + cytosine (G + C) content of DNA was calculated according to the method of Marmor and Doty [25], assuming the base composition of *S. citri* DNA to be 26 mol% G + C [28].

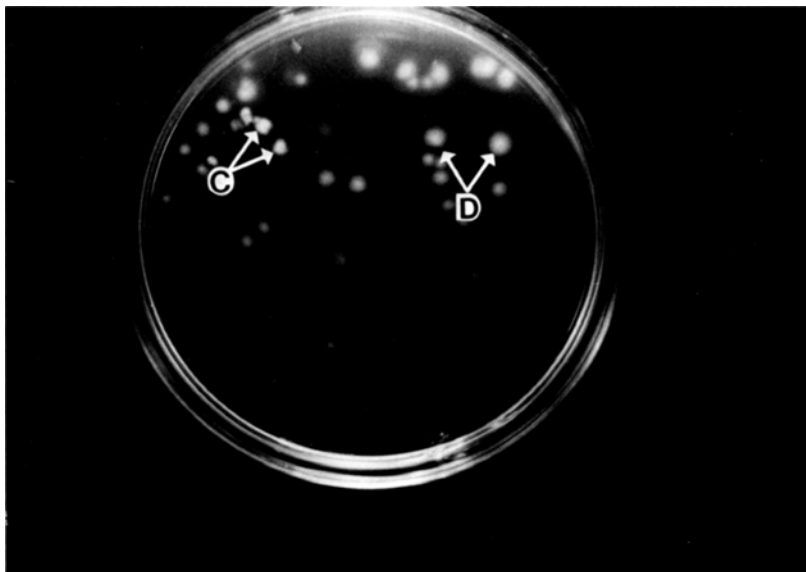


Fig. 2. Diffuse (D) and compact (C) colony forms of PPS1 spiroplasma on LD8 agar medium. Dark-field illumination.

Polyacrylamide Gel Electrophoresis (PAGE) of Cellular Proteins

Organisms were grown in broth medium LD8 for 18–20 h at 31°C, harvested by centrifugation, washed, and solubilized according to Mouches et al. [26]. Electrophoresis was performed after the method of Laemmli et al. [19] in 9% acrylamide gels with 4% acrylamide stacking gels in an Aquebogue vertical slab electrophoresis apparatus (Aquebogue Machine Shop, Aquebogue, NY 11931). In the course of preparing cultures for PAGE analyses of proteins, it was noted that colonies of PPS1 on solid (1% agar) medium LD8 were of 2 types: a diffuse colony and a rather compact colony. These colony types, designated diffuse and compact, respectively, were separated and triply cloned and were compared with one another and with strain PPS1 by PAGE analysis of proteins.

Results

Morphological Characterization

The PPS1 cell, as seen by phase contrast and dark field light microscopic observation, is a helical filament exhibiting the flexional and rotational motility typical of the spiroplasmas [14]. Transmission electron microscopy of cells, contrasted with ammonium molybdate, also revealed the helical morphology of this organism and allowed estimates of its size, ca. $0.2 \times 1\text{--}5\ \mu\text{m}$ (Fig. 1). Electron microscopy of PPS1 has previously revealed the trilaminar unit membrane, fibrillar DNA and ribosomal content, and lack of a cell wall, typical of the Mollicutes [24].

Table 3. Serological relationship between PPS1 and representative spiroplasmas of known sero-group

Antigen ^a	Serological assay	Reaction with antiserum to indicated strain					
		PPS1	SC-27	G1	CSO	23-6	SR3
SC-27	GI ^b	0	7.6	5.5	3.0	0	0
	FA ^c	0	3.0	0	— ^e	0	0
	ELISA ^d	0.03	1.0 (1.5)	0.12	0.07	0.06	0.03
G1	GI	0	10.0	10.0	4.0	0	0
	FA	0	1.0	3.0	—	0	0
	ELISA	0	0.14	1.0 (1.0)	0.03	0.01	0.01
CSO	GI	0	4.5	4.0	4.5	—	—
	ELISA	0.01	0.01	0.02	1.0 (2.2)	0.01	0.01
23-6	GI	0	0	—	—	3.9	0
	FA	0	0	0	—	3.0	0
	ELISA	0	0	0	—	1.0 (1.3)	0.01
PPS1	GI	9.8	0	0	0	0	7.0
	FA	3.0	0	0	—	0	1.0
	ELISA	1.0 (1.7)	0.01	0.04	0	0.01	0.11
SR3	GI	2.6	0	—	—	0	3.0
	FA	1.0	0	0	—	0	3.0
	ELISA	0.23	0	0.01	—	0	1.0 (1.3)
SMCA	GI	0	0	—	—	0	0

^a Abbreviations are those in Table 1.

^b GI: Growth inhibition assay as mm width of zone of growth inhibition.

^c FA: Fluorescein-conjugated antibody. Visual estimate of fluorescence of conjugated antiserum bound to indicated antigen. Scale of 0 (no fluorescence) to 3 (intense fluorescence).

^d ELISA: Values are activity relative to that of the homologous reaction. Number in parentheses after homologous reaction is the absorbance (405 nm) for the homologous reaction.

^e Assay not done.

Growth Characteristics

The PPS1 spiroplasma grows readily in both simple and complex serum-containing media such as SP-4, LD8, C3-G and MC broths. Growth was not sustained beyond 2 serial passages in serum-free SP-4 broth. PPS1 is an extremely fast-growing isolate having a doubling time of 2.5 h at 30 or 37°C, as determined by direct microscopic counts and by dilution tube assay. Time to color change of broth tubes inoculated with 1 drop of log phase culture was only 16 h at 37°C, whereas 24 h were required at 30°C. Significant growth occurred at 20°C, although the time to color change was 72 h. Populations of 10⁹ helices per ml were reached in broth media at 25–37°C.

Growth on SP-4 agar plates was accompanied by a rapid pH change evidenced by the color change of the phenol red indicator. Colonies were very difficult to observe. A very faint fogging of the medium was observed under dark field illumination and, in plates seeded with limiting dilutions of inoculum, very faint cloudy spots 3–5 mm in diameter were visible within the agar. These

Table 4. Deformation titers of 4 spiroplasmas against PPS1 antiserum

Antigen	Titer ^a
PPS1	39,366
SR3	6,561
23-6	<18
G1	<18

^a Reciprocal of dilution of PPS1 antiserum which deforms 50% of helices as observed by darkfield illumination; 3× dilution series. Normal serum gave reciprocal titers of 18 or less.

somewhat resembled the descriptions of colonies of flower spiroplasmas noted in other work [2, 7]. On LD8 agar medium in Beltsville, 2 colony types of PPS1 were observed: a diffuse colony and a rather compact colony, as noted in Figure 2.

Growth of PPS1 on agar was inhibited by digitonin as was that of *S. citri* and *Mycoplasma gallisepticum* (Table 2). As expected growth of *Acholeplasma laidlawii* was not inhibited by digitonin.

As noted in Table 2, arginine was not hydrolyzed by PPS1 since there was no secondary increase in pH at any arginine concentration tested. Arginine hydrolysis by SC-27 and CSO spiroplasmas were noted after a preliminary acid pH shift. Later, the phenol red indicator shifted back to an intense red, indicating a pH greater than 7.5–8.0.

The PPS1 spiroplasma was sensitive to oxytetracycline hydrochloride (OTC) antibiotic (Table 2). The minimum concentration inhibitory to growth of PPS1 was 0.041 µg/ml. Spiroplasmas SC-27, G1, and SR3 also had MIC values of 0.041 µg/ml. By comparison, the MIC for the 23-6 spiroplasma was 0.370 µg/ml, almost 10 times greater. The CSO strain B2 was most sensitive with an MIC of 0.013 µg/ml.

Serological Characterization

The relationships indicated by the 4 serological tests of PPS1 to other spiroplasmas representative of major serogroups are shown in Tables 3 and 4. The PPS1 strain was related to the SR3 spiroplasma [7, 11] isolated from tulip tree flowers, but heterologous reactions between PPS1 and SR3 were of a lesser magnitude in deformation, FA, and ELISA tests than were homologous reactions. There was no evidence of relatedness to any other spiroplasma group tested. Proposed separate serogroups not represented were number II of Junca et al. [18], the sex ratio organism (SRO) of *Drosophila*, and number V (flower strain brevis) of Davis and Lee [10]. The former spiroplasma has not been cultivated *in vitro*, and antisera for neither SRO nor brevis were available for our tests. However, in other work (R. E. Davis, unpublished), PPS1 antiserum gave no evidence of reaction with strain brevis in growth inhibition tests.

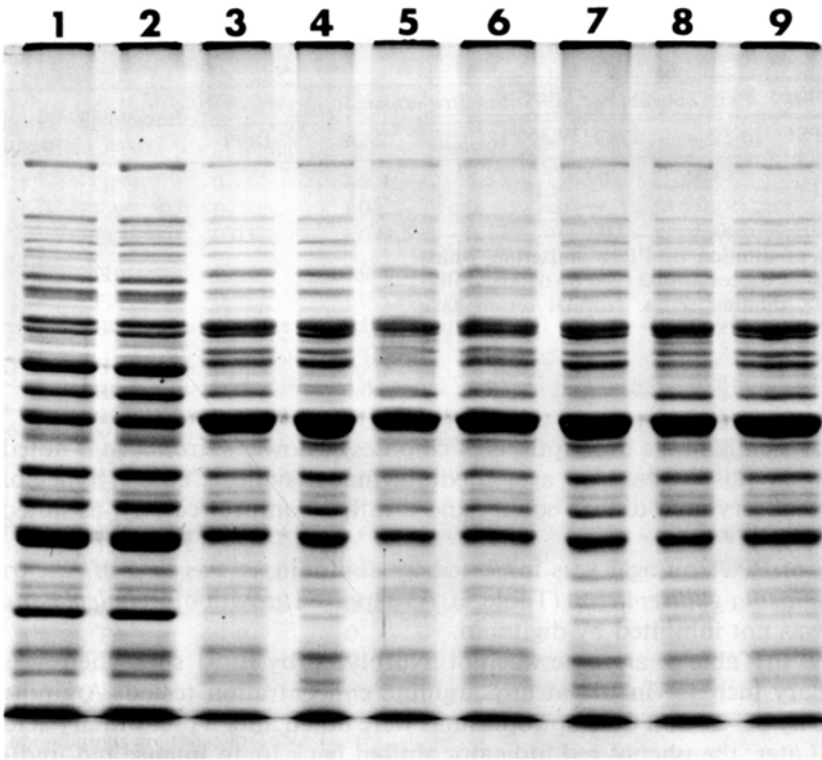


Fig. 3. Polyacrylamide gel electrophoretic patterns of proteins of SR3(1,2), PPS1 (5,8), and PPS1 clones that produce compact (4,7) and diffuse (3,6,9) colony forms.

Polyacrylamide Gel Electrophoresis of Cellular Proteins

The electrophoretic patterns of proteins from the diffuse and compact colony types of strain PPS1 were essentially identical to one another and to proteins from the original culture of strain PPS1. These patterns were similar to, but differed somewhat from the patterns of proteins from strain SR3 (Fig. 3).

Guanine + Cytosine Content of DNA

The base composition of DNA from PPS1, based on thermal denaturation, was estimated at about 29 mol% G + C, using *S. citri* as a control with a known G + C value of 26 mol% [28] (Table 5). The base composition of DNA from PPS1 is close to that estimated for flower spiroplasmas SR3 and SR9 [21] and tick spiroplasma strain SMCA [18], but is distinct from that estimated for *S. citri* [21, 28], honey bee spiroplasma AS 576 and related strains [18, 21], corn stunt spiroplasma [18, 21], tick spiroplasma 277F [18], and flower spiroplasmas of the *S. floricola* serogroup [12, 18, 21].

Table 5. Base composition of spiroplasma DNA based on thermal denaturation^a

Spiroplasma strain	T _m (°C) ^b	mol % G + C
<i>S. citri</i> (Maroc R8A2)	60.5	26.0
	60.5	26.0
Strain PPS1	61.8	29.2
	61.8	29.2

^a Two separate experiments gave identical results.

^b Melting point of extracted DNA.

Discussion

These studies demonstrate that the PPS1 spiroplasma is a unique isolate bearing a serological relationship to the SR3 spiroplasma, sufficient to include it as a member of serogroup III, as defined by Davis et al. [11], or the equivalent serogroup IV of Junca et al. [18]. The base composition of DNA from strain PPS1 (29 mol% G + C) coincides with that estimated for DNA from strain SR3 [21]. Strains PPS1 and SR3 also are similar (although not identical) to one another in pattern of proteins in PAGE analysis. These features further support the placement of strains PPS1 and SR3 in the same major spiroplasma group. However, differences between PPS1 and SR3 indicate that they may represent different subgroups of the SR3 serogroup. For example, the relatedness of PPS1 to SR3 appeared to be of about the same level as relatedness between the honey bee spiroplasma subgroup and *S. citri* subgroup representatives in the deformation, FA, and ELISA tests.

Strains of SR3 and PPS1 are clearly members of the same spiroplasma serogroup, and therefore, on the basis of criteria proposed for separation of *Spiroplasma* species [10, 11], they represent no less than a single new species distinct from *S. citri* and from spiroplasmas in other major serogroups. Although it has recently been proposed that distinct subgroups within the same serogroup can represent separate *Spiroplasma* species [10], this matter is still undecided by the International Committee on Systematic Bacteriology Subcommittee on the Taxonomy of Mollicutes. Additional studies of other members of the SR3 serogroup, such as those recently isolated in California [27], France, and Morocco [31, 33], are needed to define the phylogenetic heterogeneity of this serogroup. Should the examination of sufficient isolates of the SR3 serogroup indicate the existence of distinct strain clusters or subgroups, such as exists in the *S. citri* serogroup, strain PPS1 could be viewed as representative of a unique species distinct from SR3. Such speciation awaits recommendations by the Subcommittee on Taxonomy of Mollicutes.

The possible ecological roles played by flower-inhabiting spiroplasmas have been studied very little. However, there is no evidence to suggest that they may be plant pathogens. The ability of spiroplasmas to survive drying [29] and their frequent requirement for media of high osmolarity [2] are consistent with their survival in either the osmotically buffered nectar of the flower, or in a dried

state on the flower surface. Indeed, isolations of spiroplasmas have been made from flowers dry in appearance as well as from nectar rinsed from flowers [7].

McCoy et al. [23] and Dowell et al. [15] have demonstrated that PPS1 can multiply to high titers in hemolymph of living *Galleria mellonella* larvae and that PPS1 is highly pathogenic to this insect. Our further research will concentrate on elucidation of the possible ecological role(s) of PPS1 and other flower-inhabiting spiroplasmas and nonhelical mycoplasmas, including their potential for infecting various classes of insects and their ability to survive on the flower surface exposed to the environmental effects of desiccation, rain, heat, and solar radiation.

The existence of fastidious cell wall-less organisms on the surfaces of healthy flowers indicates an entirely new ecological niche for the mycoplasmas. It is not known whether these organisms are capable of multiplying in the nectar secreted by the flower. The flower simply may serve as a passive resting site for the organisms and as an inoculum source for flower-visiting fauna [9]. Pathogenicity of PPS1 and 2 other flower-dwelling spiroplasmas, SR3 and *S. floricola* (23-6), in *Galleria* [15], and the occurrence of the honey bee spiroplasma on flowers [8], are consistent with the possibility that insects may be the major hosts and vectors of the flower-inhabiting spiroplasmas.

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